PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

Pakistan Journal of Nutrition 15 (7): 655-661, 2016
ISSN 1680-5194

Physicochemical Properties and Amino Acid and Functional Group Profiles of Gelatin Extracted from Bovine Split Hide Cured by Acid

Dwi Wulandari[1,3], Suharjono Triatmojo[1], Yuny Erwanto[1] and Yudi Pranoto[2]
[1]Departement of Animal Product Technology, Faculty of Animal Science, Gadjah Mada University, Jl. Fauna No. 3, Bulaksumur Yogyakarta 55281, Indonesia
[2]Faculty of Agricultural Technology, Gadjah Mada University, Jl. Flora No. 1, Bulaksumur Yogyakarta, 55281, Indonesia
[3]Study Program of Leather Processing Technology, Polytechnic ATK, Jl. Ring Road Selatan, Glugo, Panggungharjo, Sewon, Bantul, Yogyakara 55188, Indonesia

Abstract: Gelatin is natural substance obtained from the partial hydrolysis of collagen from animal skin, bones and connective tissue. This study examined the influence of acetic acid concentration on characteristics of gelatin extracted from bovine split hide. Bovine split hides were obtained from a local tannery and divided into three groups cured with 0.1, 0.3, or 0.5 M acetic acid for 24 h, followed by gradient extraction at 60, 70 and 80°C for 5 h each. Data were analyzed using a completely randomized design each treatment was replicated thrice. The results showed that the acetic acid concentration significantly affected the yield, ash content, pH and gel strength, but not the moisture, fat and protein content or viscosity of the resultant gelatin. Electrophoresis of gelatin proteins showed bands distribution between 25-40 kDa. The amino acid profile of the extracted gelatin was similar to that of collagen with a high level of hydroxyproline. Infrared spectroscopy showed the presence of O-H, C = O, C = C, C-H and C-O functional groups. The results suggest that gelatin extracted from bovine split hide cured with 0.5 M acetic acid provided the best physicochemical characteristics.

Key words: Gelatin, bovine split hide, physical characteristic

INTRODUCTION

Tanning is the processing of raw animal hides into leather. During processing, one of the important stages is splitting the hide from the flesh to estimate the thickness of the leather. This leather is called the split hide and contains collagen that can then be hydrolyzed to produce gelatin (Suharjito, 2007; Hastutiningrum, 2009). Curing is an important process in the production of gelatin. Curing with acidic and basic solutions causes hydrolysis of collagen proteins in the split hide into smaller peptides. Gelatin obtained from acid curing is better than that obtained from curing with bases because acids readily digest the triple helix structure of collagen fibers into a single chain in a shorter amount of time and are often cheaper (Hasdar, 2011). Ihsanur (2010) showed that acid curing resulted in a shorter curing time and produced gelatin with a higher gel strength, greater viscosity and clearer color. Physicochemical properties of gelatin are affected by the quality of the raw hide, animal age, type of collagen present, curing and manufacturing methods, type of tissue and animal species (Gomez-Goilen *et al*., 2009; Kolodziejska *et al*., 2008).

Although application of highly concentrated curing agents can increase the quantity of gelatin obtained by increasing the amount of collagen hydrolysis, it does not guarantee a good quality product. Acid or base reactions that continuously take place at high concentrations can damage chemical bonds within collagen molecules, which reduces the quality of the resulting gelatin. Previously, Wang *et al*. (2008) showed that the acetic acid concentration affects the amount of gelatin extracted, while Ahmad and Soottawat (2011) reported that 0.2 M acetic acid influenced the characteristics of unicorn leatherjacket skin gelatin (Ahmad and Sootawat, 2011). Use of acetic acid for gelatin extraction has been reported for skin from pig (Sompie *et al*., 2015), goat (Said, 2011), fish (Binsi *et al*., 2009; Arnesen and Gildberg, 2002; Liu *et al*., 2008) and chicken legs (Ulfah, 2011). However, studies on the effect of acetic acid on the characteristics of gelatin extracted from bovine split hide are limited. Therefore, the current study investigated the effect of acetic acid concentration on the physicochemical, amino acid and functional group profile of gelatin extracted from bovine split hide.

MATERIALS AND METHODS

Materials: Bovine split hides were obtained from Magetan Tannery (East Java, Indonesia). The skins were cut into small pieces around 2750 g each. Curing was completed with acetic acid (CH3COOH), Decaltal® and distilled water.

Corresponding Author: Yuny Erwanto, Faculty of Animal Science, Gadjah Mada University, Jl. Fauna No. 3, Bulaksumur Yogyakarta 55281, Indonesia

Preparation of gelatin: Bovine split hide pieces were fleshed under dry conditions, soaked in water for 2 d, then delimed with 1% Decaltal® for 30 min. The delimed pieces were neutralized in running water to pH 6.0-7.0 and then cut into smaller pieces (1-2 cm^2) and weighed. Gelatin was prepared by type A (acid) method. Delimed bovine split hide pieces were soaked in one of three different concentrations of acetic acid (0.1, 0.3, or 0.5 M) for 24 h then neutralized with water to pH 5.0-6.0. The extraction process was performed at three different temperatures (60, 70 and 80°C) for 5 h each; filtrates were obtained by filtering with filter paper (0.45 µm). In order to increase the concentration of gelatin extracted, filtrates were incubated in a waterbath at 60°C for 5 h then poured in a tray and dried in an oven at 50°C for 36-48 h. Gelatin sheets were milled and packaged in plastic bottles and stored in a desiccator for subsequent processing.

Physicochemical, protein, pH and viscosity analyses: The physicochemical properties of the extracted gelatin (yield, moisture, fat and ash content) were determined by Association of Analytical Communities (AOAC, 2005) methods. Protein content was determined using the Kjeldahl method with conversion factors of 5,5. pH was determined by pH meter (Hanna Instrument 9043 screw type electrode). Viscosity was measured by Behlin CSR-10 Stromer viscometer. Gelatin powder was completely dissolved in distilled water at 60°C with stirring to a concentration of 6.67% (w/v) and viscosity was expressed in centipoise [cP] (Arnesen and Gilberg, 2005).

Gel strength: Gel strength was determined according to methods of Muyonga *et al.* (2004) and Liu *et al.* (2008) using a Zwick Z0.5 Universal Testing Machine. Gelatin solutions [6.67% (w/v)] were placed in containers (diameter, 5 cm; height, 6 cm) at 5°C for 16-18 h, before being placed at the bottom of the Testing Machine plunger (diameter, 13 mm). Measurements were taken at 10°C with a plunger speed of 10 mm/min and 4-mm depth. Gel strength (in Bloom) was equivalent to the maximum force (in g), which was equivalent to (g/mm^2) x 12.7 mm^2 (the surface area of the needle).

Distribution of molecular weight: The molecular weight distribution is determined according to the Laemmli (1970) method with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gelatin samples were diluted with sterile distilled water, mixed, then centrifuged at 3000 ppm for 10 min at room temperature. Samples and supernatants were mixed 1:1, denatured at 100°C for 2 min and then cooled at room temperature. Gelatin samples (10 µL) were loaded onto a 12% running gel with a 5% stacking gel. After electrophoresis, gels were stained with Coomassie Blue.

Amino acid profiles: The amino acid profile of gelatin samples was determined by high performance liquid chromatography according to a previous method (Games, 1987).

Functional group profile: Gelatin sample functional groups were analyzed using a 96600 Frontier Fourier Transform Infrared (FTIR) Spectroscope (Perkin Elmer, Inc., USA) equipped with a universal attenuated total reflectance diamond/ZnSe crystal at room temperature. Gelatin sample FTIR spectra were in the 500-4000 per cm range and collected in four scans with automatic signal gain control at a resolution of 16 per cm against a background spectrum recorded from a clean, empty cell. FTIR was conducted with a mid-IR deuterated triglycine sulfate detector and optical KBr beam splitter.

Experimental design and data analysis: Data were analyzed using a completely randomized design, if that significant followed by Duncan's Multiple Range test. Bovine split hide samples were cured each acetic acid concentration in triplicate. Descriptive analysis was used for molecular weights, amino acids and functional groups. Data obtained from bovine split hide gelatin were then compared to commercial gelatin.

RESULTS AND DISCUSSION

Physicochemical properties of gelatin extracted from bovine split hide: The physicochemical properties of gelatin extracted from bovine split hide are shown in Table 1. Treatment with different acetic acid concentrations had no effect on the water content of extracted gelatin, which was similar to the Indonesian National Standard [16%] (Anonimous, 2005). Although 0.5 M acetic acid had no effect on gelatin water content in the current study, at high enough concentrations, inter- and intramolecular collagen bonds stretch and break, accelerating the process of denaturation and eventually impacting the water content of the resultant gelatin (Said, 2011). In contrast, the protein content increased with increasing acetic acid concentration (Table 1); high concentrations of curing agents reportedly augment denaturation of collagen proteins (Said, 2011). Furthermore, Choi and Regenstain (2000) indicated that gelatin protein content is affected by the type, quality and quantity of the raw materials.

The fat content in extracted gelatin did not exceed 5% (the maximum limit of quality) required for with any concentration of acetic acid (Table 1). During the curing process, proteins denature (Zeugollis *et al.*, 2008) and dissolve into solution (Wang *et al.*, 2008). Partial and complete denaturation of proteins releases any associated fat molecules which also undergo a process of dissolution and washing wasted when post-curing. Duncan's Multiple Range test showed acetic acid concentration affected the ash content of gelatin extracted from bovine split hide. The results showed that the higher the concentration of acetic acid, the lower the ash content. This was likely due to acetate acid as a marinade solution was an organic acid that did not contain minerals that all burned when ashed (Ulfah, 2011).

The pH of extracted gelatin was also affected by the acetic acid concentration. Table 1 shows that a higher

Table 1: Physicochemical properties of bovine split hide gelatin

Physical and chemical properties	Curing in acetic acid		
	T1 (0.1 M)	T2 (0.3 M)	T3 (0.5 M)
Moisture (%)	6.48[a]	6.82[a]	6.28[a]
Protein (%)	52.22[a]	67.15[a]	69.07[a]
Fat (%)	0.26[a]	0.40[a]	0.32[a]
Ash (%)	0.84[a]	0.53[a]	0.3[b]
pH	6.53[a]	5.81[b]	5.41[c]
Yield (%)	5.95[a]	6.99[a]	9.2[b]
Viscosity (cp)	6.48[a]	6.48[a]	6.88[a]
Gel strength (Bloom)	22.95[a]	149.64[b]	153.53[b]

[a,b]Different superscripts in the same row indicate significant differences ($p<0.5$)

Table 2: Analysis of amino acid composition in gelatin extracted from bovine split hide

Amino acid	T1	T2	T3	Reference
Aspartic acid	3.4	3.2	3.9	4.6*
Glutamic acid	6.6	6.1	7.5	7.4*
Serine	2.3	2.3	2.8	3.9*
Histidine	1.2	1.2	1.3	0.4*
Glycine	10.1	10.1	11.3	34.2*
Arginine	5.2	4.6	6.2	4.7*
Alanine	5.6	5.6	6.9	11.3*
Tyrosine	1.0	1.1	1.1	0.4*
Methionine	1.1	0.4	1.2	0.4*
Valine	1.6	1.5	1.7	1.9*
Phenylalanine	1.7	1.6	1.8	1.2*
Isoleucine	1.3	1.3	1.4	1.5**
Leucine	2.2	2.0	2.3	2.4*
Lysine	2.4	2.4	3.0	2.5*
Cysteine[a]	2.5	1.6	1.8	-
Proline[a]	15.7	11.5	12.3	12.4**
Hydroxyproline[a]	25.15	18.33	21.49	11.9**

T1, T2 and T3 = gelatin extracted with 0.1, 0.3 and 0.5 M acetic acid. [a]Spectrophotometric analysis

*Amino acid content of bovine skin gelatin reported by Gomes Estaca *et al*. (2008)

**Amino acid content according to GMAP (2006)

concentration of acetic acid directly correlated with a lower pH. This was likely the result of curing with an acid. During the soaking process, collagen fibers in the skin swell due to diffusion of acetic acid into the tissue. Then, the acid leaching process that remains in the skin more (Ulfah, 2011; Peranginangin, 2004).

Yield: Curing with different concentrations of acetic acid significantly affected ($p<0.05$) the gelatin yield. Diffusion of acetic acid into the skin breaks covalent crosslinkages between collagen molecules, enabling more hydrolysis of collagen protein into gelatin and better dissolution of product in the extraction stage (Handoko *et al*., 2011). A higher concentration of acid means an increase in H^+ ions that ultimately accelerate the process of hydrolysis, thereby increasing the number of collagen molecules converted to gelatin and increasing the yield (Zhou and Joe, 2005). Moreover, acid treatment can remove non-collagen proteins and extraction with hot water can cause

Table 3: Analysis of functional groups on bovine split hide gelatin using Fourier Transform Infrared spectroscopy

Wave number at the peak absorption (per cm)	Estimate functional group	Name
T1 (acetic acid 0.1M)		
3268.91	O - H	Carboxylic acid
1630.60	C = O	Amide
1522.68	C = C	Aromatic
1398.63	C - H	Alkane
1239.75	C - O	Ester
T2 (acetic acid 0.3 M)		
3275.90	O - H	Carboxylic acid
1626.97	C = O	Amide
1522.74	C = C	Aromatic
1438.41	C - H	Alkane
1233.81	C - O	Ester
T3 (acetic acid 0.5 M)		
2969.91	O - H	Carboxylic acid
1739.23	C = O	Imide
1621.36	C = C	Aromatic
1369.89	C - H	Alkane
1220.73	C - O	Ester
Gelatin commercial (Merk)		
3274.92	O - H	Carboxylic acid
1628.26	C = O	Amide
1524.12	C = C	Aromatic
1230.27	C - O	Ester

T1, T2 and T3 = gelatin extracted with 0.1, 0.3 and 0.5 M acetic acid

further decomposition of the collagen structure and produce smaller gelatin molecules (Cheow *et al*., 2007).

Viscosity: Viscosity is defined as the internal friction or drag that affects the flow of fluids (Andarwulan *et al*., 2011). Though the average viscosity of bovine split hide gelatin increased slightly with 0.5 M acetic acid (Table 1), this change was not significant ($p>0.05$). This increase may have been caused by higher concentrations of H^+ ions breaking peptide bonds within collagen molecules, whereas 0.1 and 0. 3 M acetic acid were not concentrated enough to completely break these bonds and produced a lower viscosity.

Gel strength: Statistical analysis showed that the concentration of acetic acid used in the curing process significantly affected the gel strength ($p<0.05$, Table 1). On average, gel strength increased with increasing acetic acid concentration. Higher concentrations of acid are able to hydrolyze more collagen into gelatin, there by increasing resultant gel strength. Gel strength is related to hydrogen bonds between water molecules and free O-H groups on amino acids, protein chain size, concentration and molecular weight distribution of collagen proteins (Karim and Bhat, 2008). Average of gel strength values obtained ranged from 22.95 to 153.53 Bloom. Similar results were

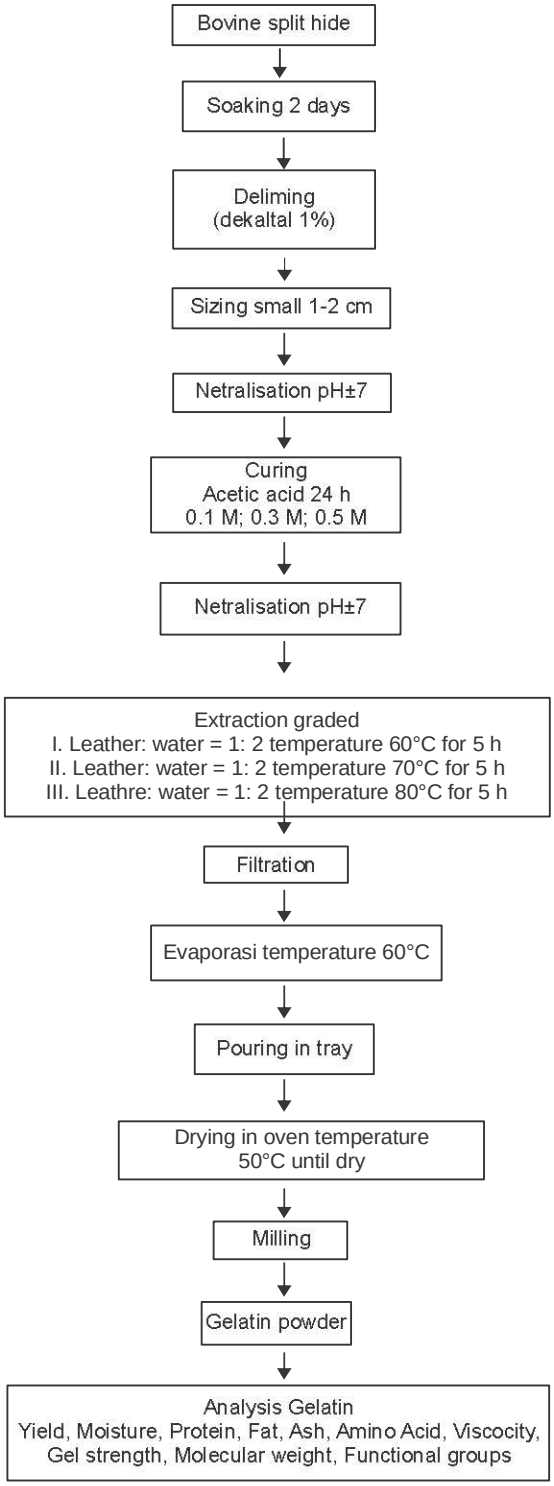

Fig. 1: Flowchart of gelatin extraction from bovine split hide (adapted from Suharjito, 2007)

also found by Widayanti (2014) for gelatin extracted from bovine hides using a basic curing agent (22.78-129.38 Bloom).

Molecular weight distribution of gelatin proteins: Interestingly, gelatin proteins extracted from bovine split hide by 0.3 and 0.5 M acetic acid showed the same banding pattern, whereas 0.1 M acetic acid was devoid of bands altogether (Fig. 2). This was probably due to the fact that 0.1 M acetic acid was unable to successfully fragment collagen into small enough pieces able to traverse the polyacrylamide gel. Commercial gelatin showed the same protein bands between 25-40 kDa as 0.3 and 0.5 M acetic acid, but also had additional higher molecular weight bands. The molecular weight distribution of gelatin proteins is suggested to be most closely related to the level of long-chain amino acids present and gel strength (Badii and Nazlin, 2006). In other words, proteins with large molecular weights confer greater gel strength. Furthermore, the molecular weight of gelatin proteins is largely responsible for its functional properties and the behavior of gels (Muyonga *et al*., 2004).

Amino acid profile: Table 2 shows how the amino acid composition of bovine split hide gelatin was affected by the concentration of curing agent. The acid curing process denatures some specific amino acids, thereby altering the amino acid composition of gelatin proteins present (Abustam *et al*., 2003). Collagen protein has a high concentration of glycine, proline and hydroxyproline, but very small amounts of aromatic and sulfur-containing amino acids (Wolf, 2003). Hence, differences in the amino acid composition of gelatin protein products affect the physical quality, strength, viscosity and melting point of the gel (Arnesen and Gildberg, 2005).

Functional group profile: The functional groups are special groups of atoms in a molecule that underlay the characteristic chemical reactivity of each molecule (Said, 2011). Figure 3 and Table 3 show that curing bovine split hide with different concentrations of acetic acid and commercial gelatin have similar FTIR spectra but different absorption peak intensities. This means that the functional groups detected in extracted gelatin proteins are relatively similar to those within commercial gelatin, suggesting identical functional properties between the two types of gelatin. Thus, bovine split hide gelatin could be a potential substitute for commercial gelatin since compounds that have similar functional groups tend to have similar chemical reactivity (Anonimous, 2009).

FTIR spectra for gelatin extracted from bovine split hide formed five characteristic peaks corresponding to carboxylic acid (O-H, 2500-360 per cm), amide (C = O, 1690-1630 per cm), aromatic C = C bonds (1650-1450 per cm), alkanes (C-H groups; 1470-1350 per cm) and esters (C-O, 1300-1000 per cm). The different absorption peak intensities of gelatin proteins produced by curing with different concentrations of acetic acid denote their different physical and/or chemical properties. Functional groups containing detectable, function besides derived from the

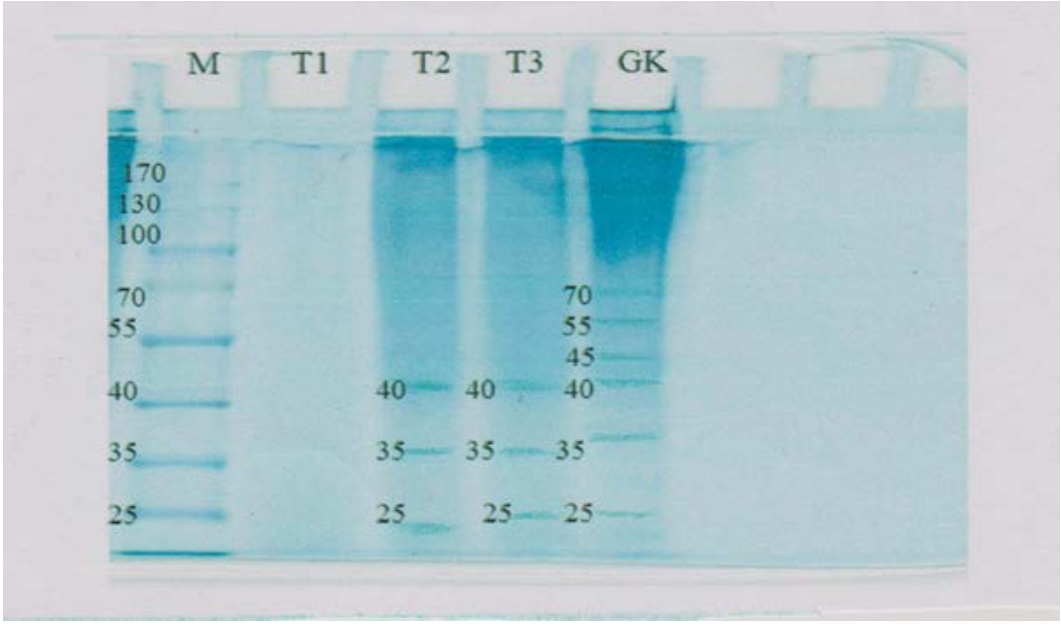

Fig. 2: SDS-PAGE of bovine split hide gelatin. M = marker; T1, T2 and T3 = gelatin extracted with 0.1, 0.3 and 0.5 M acetic acid; GK= commercial gelatin (Merk)

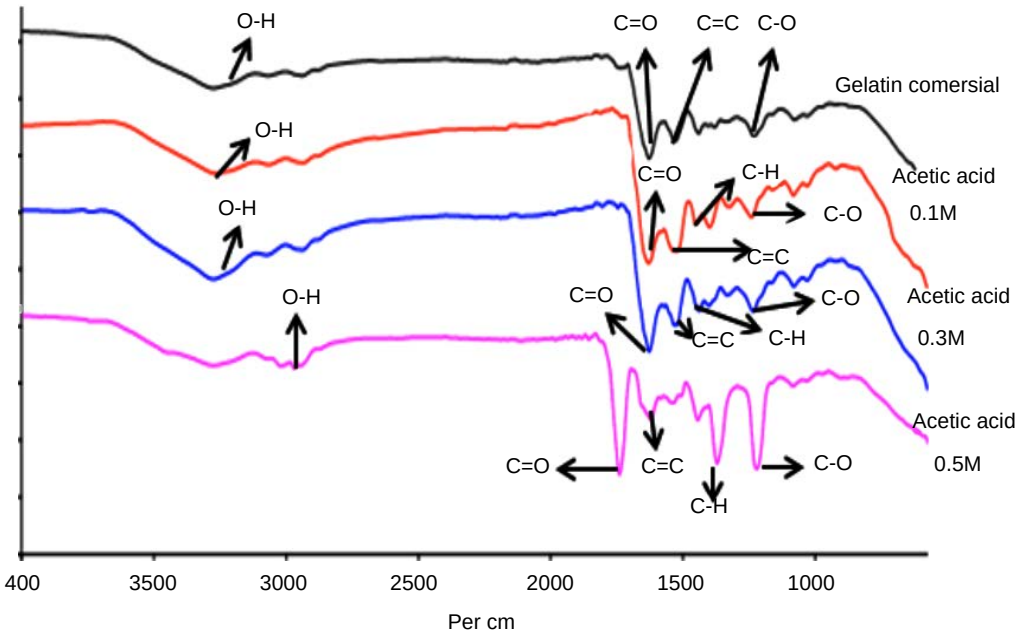

Fig. 3: Fourier Transform Infrared spectra of gelatin extracted from bovine split hide

amino acid structure the possibility can be derived from curing agent used (Sastrohamidjojo, 1992). For example, absorption widened at 3268, 3275, 2969 and 3274 per cm for gelatin extracted with 0.1, 0.3 and 0.5 M acetic acid and commercial gelatin, respectively. Wide peaks are characteristic of O-H groups on hydroxyproline (Puspawati *et al*., 2012). Most peaks corresponding to free N-H groups between 3650 and 3580 per cm are narrow and sharp. If the N-H group of a peptide hydrogen bonds, the corresponding peak will shift to a lower wave number and N-H overlap with nearby O-H groups causes peak widening.

Peaks at 1630, 1626, 1739 and 1628 per cm produced by gelatin extracted by 0.1, 0.3 and 0.5 M acetic acid and

commercial gelatin, respectively, correspond to an absorption area where C = O and O-H groups compete with carboxyl group absorption. This area is known as for residual imide absorption. Subsequent uptake is aromatic C = C groups at 1522, 1621 and 1524 per cm for commercial gelatin. Peaks at 1398, 1438 and 1369 per cm correspond to C-H groups in gelatin extracted with 0.1, 0.3 and 0.5 M acetic acid, while that for commercial gelatin was not visible because of O-H peak overlap. The last absorption peak area from 1000-1300 per cm is due to vibration of C-O groups of short-chain peptides that occur due to degradation of the peptide chain (Jackson, 1995). Interestingly, N-H functional groups characteristic of all proteins are not apparent in the absorption spectrum (Fig. 3). This likely occurred because the absorption intensity of these N-H groups (3500-300 per cm) overlap with that of O-H groups (3650-3200 per cm), producing a very wide ribbon shape (Sastrohamidjojo, 1992).

Conclusion: Physicochemical analysis, molecular weight distribution, as well as amino acid and functional group profiles of gelatin extracted from bovine split hide showed that curing with different concentrations of acetic acid produced similar characteristics.

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